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Quantification of the Major Urinary Metabolite of 15-F_{2t}-Isoprostane (8-iso-$PGF_{2\alpha}$) by a Stable Isotope Dilution Mass Spectrometric Assay

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The isoprostanes (IsoPs) are a series of novel prostaglandin (PG)-like compounds generated from the free radical-catalyzed peroxidation of arachidonic acid. The first series of IsoPs characterized contained F-type prostane rings analogous to $PGF_{2\alpha}$. One F-ring IsoP, 15-F_{2t}-IsoP (8-iso-$PGF_{2\alpha}$) has been shown to be formed in abundance *in vivo* and to exert potent biological activity. As a means to assess the endogenous production of this compound, we developed a method to quantify the major urinary metabolite of 15-F_{2t}-IsoP, 2,3-dinor-5,6-dihydro-15-F_{2t}-IsoP (2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$), by gas chromotography/negative ion chemical ionization mass spectrometry. This metabolite was chemically synthesized and converted to an $^{18}O_2$-labeled derivative for use as an internal standard. After purification, the compound was analyzed as a pentafluorobenzyl ester trimethylsilyl ether. Precision of the assay is ±4% and accuracy is 97%. The lower limit of sensitivity is approximately 20 pg. Levels of the urinary excretion of this metabolite in 10 normal adults were found to be 0.39 ± 0.18 ng/mg creatinine (mean ± 2 SD). Substantial elevations in the urinary excretion of the metabolite were found in situations in which IsoP generation is increased and antioxidants effectively suppressed metabolite excretion. Levels of 2,3-dinor-5,6-dihydro-15-F_{2t}-IsoP were not affected by cyclooxygenase inhibitors. Thus, this assay provides a sensitive and accurate method to assess endogenous production of 15-F_{2t}-IsoP as a means to explore the pathophysiological role of this compound in human disease.

Key Words: **isoprostane; eicosanoid; prostaglandin; metabolite; oxidation; lipid.**

Free radicals derived primarily from oxygen have been implicated in the pathogenesis of a wide variety of human disorders (1–4). One of the major targets of free radical injury is lipids, which undergo peroxidation. Previously, we reported that a series of prostaglandin (PG)[1] F_2-like compounds, termed F_2-isoprostanes (F_2-IsoPs), are produced *in vivo* as products of the free radical-catalyzed peroxidation of arachidonic independent of the cyclooxygenase (5). Four positional isomers of the F_2-IsoPs are formed, each of which can be composed of eight racemic diastereomers. Generation of compounds with side chains oriented *cis* in relation to the prostane ring are highly favored over *trans* side chain-containing compounds (6). In this respect, we have previously demonstrated that 15-F_{2t}-IsoP (8-iso-$PGF_{2\alpha}$) is one of the more abundant F_2-IsoPs produced *in vivo* (7). There has been considerable interest in this molecule because it exerts potent biological activity, e.g., it is a potent vasoconstrictor in the lung and kidney (8). Furthermore, it has been suggested that the biological effects of 15-F_{2t}-IsoP may result from interaction with a unique receptor.

It has been recognized that one of the greatest impediments in the field of free radical research has been the lack of reliable methods to assess oxidant stress status in humans (9). A large body of evidence has been accumulated indicating that the measurement of F_2-IsoPs provides a valuable and accurate approach to

[1] Abbreviations used: PG, prostaglandin; IsoP, isoprostane; GC, gas chromatography; NICI, negative ion chemical ionization; MS, mass spectrometry; SIM, selected ion monitoring; NMR, nuclear magnetic resonance; BSTFA, *N,O*-bis(trimethylsilyl)trifluoroacetamide; SIM, selected ion monitoring.

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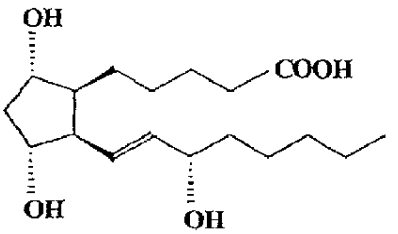

FIG. 1. Structure of the major urinary metabolite of 15-F_{2t}-IsoP, 2,3-dinor-5,6-dihydro-15-F_{2t}-IsoP (15-F_{2t}-IsoP-M).

assess oxidant stress *in vivo* both in animal models of oxidant injury and in humans (8). In this regard, however, the quantification of unmetabolized IsoPs has certain limitations. First, F_2-IsoPs can be artifactually generated *ex vivo* in biological fluids, such as plasma, by autoxidation of plasma arachidonic acid if appropriate precautions are not taken (6). In addition, quantification of F_2-IsoPs esterified in tissues or circulating in plasma provides information about oxidant stress status only at a particular time or in selected organs rather than an integrated index of IsoP production (8). Having a means to obtain an integrated index of oxidant stress status would be extremely valuable in situations in which the level of oxidative injury fluctuates over time. Thus, analogous to quantification of urinary metabolites of cyclooxygenase-derived prostanoids, measurement of the urinary excretion of F_2-IsoPs should provide a reliable and integrated index of oxidative stress status *in vivo* (10).

With this goal in mind, we have previously carried out studies examining the metabolic fate of 15-F_{2t}-IsoP in humans and determined that the major urinary urinary metabolite was 2,3-dinor-5,6-dihydro-15-F_{2t}-IsoP (2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$) (Fig. 1) (11). The chemical synthesis of this metabolite, hereafter termed 15-F_{2t}-IsoP-M, was subsequently undertaken to enable the development of a mass spectrometric assay based on stable isotope dilution methodology. The method of assay that has been developed is described herein.

MATERIALS AND METHODS

Reagents. Pentafluorobenzyl bromide and diisopropylethylamine were obtained from Sigma Chemical Co. (St. Louis, MO). Dimethylformamide, undecane, and 1-butane boronic acid were obtained from Aldrich Chemical Co. (Milwaukee, WI). *N,O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) was obtained from Supelco Inc. (Bellefonte, PA). All organic reagents were obtained from Allied Signal (Burdick and Jackson Brand, Muskegon, MI). C_{18} and silica Sep-Paks were purchased from Waters Associates (Milford, MA). TLC was performed on silica gel 60ALK6D plates (Whatman International Ltd., Maidstone, UK).

Mass spectrometry. GC/NICI MS was performed using a Hewlett–Packard HP5989A GC/MS instrument interfaced with an IBM Pentium II computer system. GC was performed using a 15-m, 0.25-mm-diameter, 0.25-μm-film thickness, DB1701 fused silica capillary column (Fisons, Folsom, CA). The column temperature was programed from 190 to 300°C at 15°C/min. Methane was used as the carrier gas at a flow rate of 1 ml/min. Ion source temperature was 250°C, electron energy was 70 eV, and filament current was 0.25 mA. For analysis, compounds were dissolved in 10 μl of undecane which was dried over a bed of calcium hydride.

Statistical analyses. Data were analyzed using Student's *t*-test. Differences were considered significant if $P < 0.05$.

Preparation of $^{18}O_2$-labeled 15-F_{2t}-IsoP-M. Unlabeled 15-F_{2t}-IsoP-M was chemically synthesized by one of us (D.T.) using previously described methods (12). The structure of 15-F_{2t}-IsoP-M was confirmed using MS and NMR. Unlabeled 15-F_{2t}-IsoP-M was subsequently converted to an $^{18}O_2$-labeled derivative for use as an internal standard by the method of Murphy *et al.* involving successive steps of methylation and alkaline hydrolysis with $Li^{18}OH$ (13). This yielded an internal standard with an unlabeled blank of 3 parts per 1000 when analyzed by GC/NICI MS. The blank remained unaltered when the labeled standard was subjected to the assay procedure subsequently developed. The $^{18}O_2$-labeled 15-F_{2t}-IsoP-M was standardized utilizing GC/NICI MS against unlabeled 15-F_{2t}-IsoP-M that had been accurately weighed.

Purification and analysis of urinary 15-F_{2t}-IsoP-M. Figure 2 summarizes the purification and derivatization scheme for the analysis of 15-F_{2t}-IsoP-M. To 0.25 ml of urine is added 1.5 ng of the [$^{18}O_2$]15-F_{2t}-IsoP-M internal standard. The sample is then acidified to pH 3 with 1 M HCl and diluted to 3 ml with H_2O. The mixture is vortexed and applied to a C_{18} Sep-Pak column preconditioned with 5 ml methanol and 5 ml of water (pH 3). The sample and subsequent solvents are eluted through the Sep-Pak using a 10-ml plastic syringe. The column is then washed sequentially with 10 ml of water (pH 3) and 10 ml heptane. 15-F_{2t}-IsoP-M is eluted with 10 ml ethyl acetate/heptane (50:50, v/v).

The ethyl acetate/heptane eluate from the C_{18} Sep-Pak is then dried over anhydrous Na_2SO_4 and applied to a silica Sep-Pak. The cartridge is then washed with 5 ml of ethyl acetate followed by elution of 15-F_{2t}-IsoP-M with 5 ml of ethyl acetate/methanol (50:50, v/v). The ethyl acetate/methanol eluate is evaporated under a stream of nitrogen.

15-F_{2t}-IsoP-M is then converted to a pentafluorobenzyl ester by treatment with a mixture of 40 μl of 10% pentafluorobenzyl bromide in acetonitrile and 20 μl of 10% *N,N*-diisopropylethylamine in acetonitrile at room

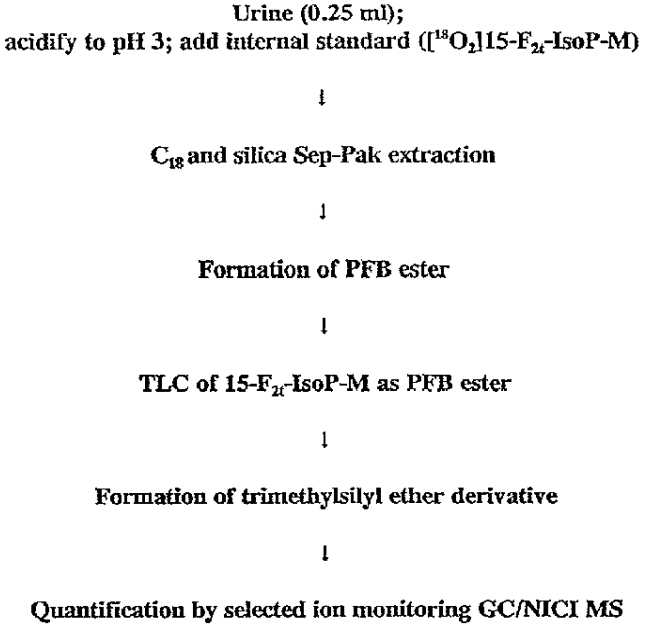

FIG. 2. Scheme for the purification, derivatization, and analysis of 15-F_{2t}-IsoP-M.

temperature for 30 min. The reagents are dried under nitrogen and this procedure is repeated to ensure quantitative esterification. After the second esterification, the reagents are dried under nitrogen and the residue subjected to TLC using the solvent ethyl acetate/methanol (98:2, v/v). Approximately 2–5 μg of the methyl ester of $PGF_{2\alpha}$ is chromatographed on a separate lane and visualized by spraying with a 10% solution of phosphomolybdic acid in ethanol followed by heating. Compounds migrating in the region of the methyl ester of $PGF_{2\alpha}$ (R_f 0.22) and the adjacent area 1.1 cm above are scraped and extracted from the silica gel with ethyl acetate.

The ethyl acetate is dried under nitrogen and 15-F_{2t}-IsoP-M is then converted to a trimethylsilyl ether derivative by adding 20 μl BSTFA and 10 μl dimethylformamide and incubating at 40°C for 20 min. The reagents are dried under nitrogen and 15-F_{2t}-IsoP-M is redissolved in 10 μl of undecane for analysis by GC/MS.

The major ion generated in the NICI mass spectrum of the pentafluorobenzyl ester, tris trimethylsilyl ether derivative of 15-F_{2t}-IsoP-M, is *m/z* 543, which represents the M-181(M-$CH_2C_6F_5$) carboxylate anion. The $^{18}O_2$-labeled internal standard generates an analogous ion at *m/z* 547. Quantification of endogenous 15-F_{2t}-IsoP-M is accomplished by SIM analysis of the ratios of intensities of *m/z* 543 to *m/z* 547. The recovery of 15-F_{2t}-IsoP-M through the assay is approximately 20–30%. Two steps account for 90% of the losses of material through the assay. They are the Sep-Pak extractions (approximately 30% loss) and TLC (approximately 50% loss).

RESULTS

Assay results. A representative SIM chromatogram obtained from the analysis of urine from a normal human is depicted in Fig. 3. At the bottom is the *m/z* 547 SIM chromatogram representing the $^{18}O_2$-labeled internal standard. In the upper *m/z* 543 chromatogram, several peaks are present. The starred (*) peak directly above the internal standard represents endogenous 15-F_{2t}-IsoP-M. This is supported by a number of observations, including the fact that material present in this *m/z* 543 peak contains the same functional groups (three hydroxyls, no carbonyls) and number of double bonds (2) that are present in chemically synthesized 15-F_{2t}-IsoP-M (11). In addition, the stereochemical orientation of the cyclopentane ring hydroxyls of this compound was determined and, like 15-F_{2t}-IsoP, it possessed hydroxyls that were exclusively oriented *cis* since it formed a boronate derivative after reaction with *n*-butane boronic acid (data not shown) (6). Furthermore, as discussed subsequently, levels of this compound generated *in vivo* are altered by changes in redox status in humans and animals. The amount of 15-F_{2t}-IsoP-M represented by the starred (*) peak in the *m/z* 543 chromatogram (Fig. 2) is 0.43 ng/ml urine.

Although the identification of the other chromatographic peaks present in the *m/z* 543 chromatogram has not been entirely elucidated, we have found that they possess the same functional groups and number of double bonds as 15-F_{2t}-IsoP-M and also have ring hydroxyls that are in the *cis* configuration. Thus, it is likely that since a number of F_2-IsoPs are formed *in vivo*, compounds represented by these other *m/z* 543 chromatographic peaks are metabolites of other IsoPs that have structures analogous to 15-F_{2t}-IsoP-M. Further studies are underway to confirm this hypothesis.

Assay parameters and validation. Quantification of the 15-F_{2t}-IsoP-M is based on the intensity of the starred (*) peak shown in Fig. 3. The lower limit of detection (signal to noise ratio of approximately 4:1) of 15-F_{2t}-IsoP-M is in the range of 20 pg. Several procedures were performed to establish the accuracy of this assay. Initially, a standard curve was constructed by adding varying amounts of unlabeled 15-F_{2t}-IsoP-M to a fixed quantity of 1.5 ng of [$^{18}O_2$]15-F_{2t}-IsoP-M and the measured ratio of *m/z* 543 to *m/z* 547 to the expected ratio compared (Fig. 4). The standard curve was found to be linear over a 25-fold concentration range.

Experiments were then carried out to establish the precision and accuracy of the assay. Precision was measured by analyzing six 1-ml aliquots of urine obtained in a 24-h collection from a normal volunteer. The mean of three replicate measurements of the ratio

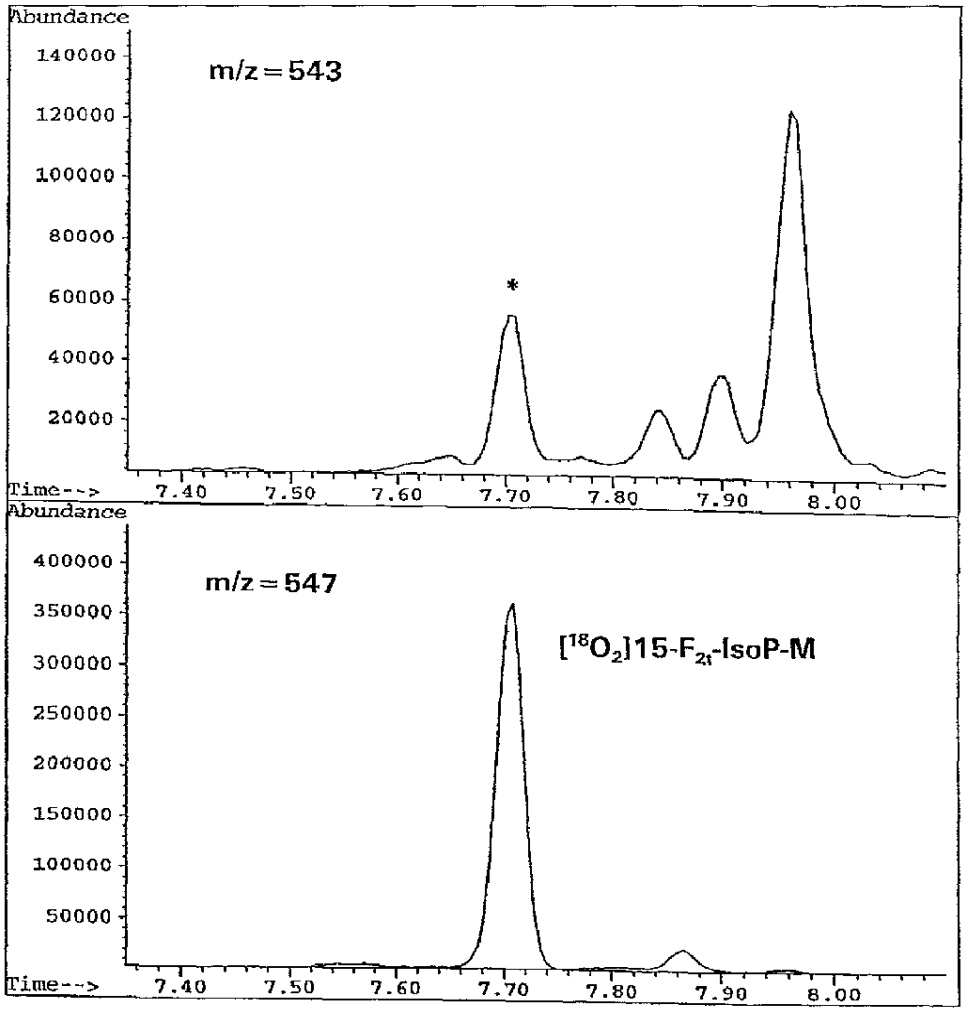

FIG. 3. Analysis of urine from a normal human for 15-F_{2t}-IsoP-M using GC/MS.

of *m/z* 543 to *m/z* 547 was found to be ±4%. Accuracy was assessed using the same urine. For this, 1.0 ng of unlabeled 15-F_{2t}-IsoP was added to another four 1-ml aliquots of the urine and reassayed. The amount of endogenous 15-F_{2t}-IsoP-M measured in the precision experiment was subtracted from the total measured, and the accuracy of the assay to measure the added 1.0 ng of 15-F_{2t}-IsoP-M was calculated. The accuracy was found to be 97%.

The effect of storage of urine on levels of 15-F_{2t}-IsoP-M was also investigated. In two urine samples from each of three individuals, there was less than a 7% variation in the level of 15-F_{2t}-IsoP-M measured in the urine that was analyzed immediately compared to the level measured following storage at −70°C for 6 months.

Urinary 15-F_{2t}-IsoP-M levels in normal humans. To establish the normal range of the urinary excretion of 15-F_{2t}-IsoP-M, aliquots of urine from 24-h urine collections were obtained and analyzed from five healthy men and five healthy women (ages 20–40 years old). Normal levels were found to be 0.39 ± 0.18 ng/mg creatinine (mean ± 2 SD). There were no significant differences in the levels of 15-F_{2t}-IsoP-M in males versus females (men, 0.39 ± 0.20 ng/mg creatinine versus women, 0.39 ± 0.16 ng/mg Cr). In addition, in three individuals administered high dosages of either of the cyclooxygenase inhibitors aspirin (2500 mg/day) or ibuprofen (3200 mg/day), urinary levels of 15-F_{2t}-IsoP-M were not significant different compared to those before treatment (0.42 ± 0.22 ng/mg creatinine after treatment versus 0.36 ± 0.14 ng/mg creatinine before).

Urinary F_{2t}-IsoP-M levels in situations associated with altered F_2-IsoP generation. We have examined the ability of the assay to assess the overproduction of 15-F_{2t}-IsoP-M in clinical situations in which increased

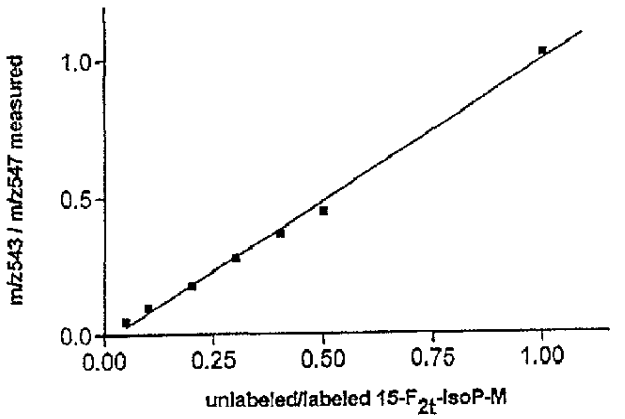

FIG. 4. Standard curve for the analysis of 15-F_{2t}-IsoP-M by GC/MS. Varying quantities of unlabeled 15-F_{2t}-IsoP-M were added to 1.5 ng of [$^{18}O_2$]15-F_{2t}-IsoP-M to give the ratios noted on the horizontal axis. On the vertical axis are plotted the actual ratios of *m/z* 543 (unlabeled 15-F_{2t}-IsoP-M) to *m/z* 547 (labeled 15-F_{2t}-IsoP-M) measured by selected ion monitoring analysis. The linear equation describing the standard curve is $y = 1.01x - 0.02$.

quantities of F_2-IsoPs are known to be generated *in vivo*. Patrono and colleagues have reported that 15-F_{2t}-IsoP is significantly overproduced in humans with hypercholesterolemia (14). Therefore, we quantified 15-F_{2t}-IsoP-M in urine collections from five individuals with polygenic hypercholesterolemia (serum cholesterol > 250 mg/dl). As shown in Fig. 5, levels of 15-F_{2t}-IsoP-M are significantly increased (mean, 2.5-fold) in hypercholesterolemic individuals compared to controls with normal cholesterol levels ($P < 0.05$). Levels of plasma free F_2-IsoPs were also significantly higher in the hypercholesterolemic group.

It has been previously been shown by FitzGerald and other investigators that antioxidants decrease endogenous IsoP formation in humans (14, 15). To determine whether this is also the case for 15-F_{2t}-IsoP-M, we administered the five hypercholesterolemic patients noted above high doses of the antioxidants vitamin C (8 g/day), vitamin E (3200 IU/day), and β-carotene (240 mg/day). After 8 weeks of therapy, urinary 15-F_{2t}-IsoPs were again quantified. As shown in Fig. 5, after receiving antioxidant therapy, levels of the metabolite were significantly decreased a mean of 54% ($P < 0.05$ compared to pretreatment levels). Plasma free F_2-IsoP levels also decreased a mean of 35%. These data confirm that antioxidants modulate endogenous IsoP formation in humans.

We have also examined the formation 15-F_{2t}-IsoP-M in rats administered CCl_4 (1 ml/kg) orogastrically to induce an oxidant stress. In this model, levels of F_2-IsoPs in tissues and plasma increase dramatically after treatment (8). For this study, urine was collected from each of four animals 12 h prior to and 12 h after administration of CCl_4. Although metabolism of eicosanoids can vary widely among different animal species (16), we have found that basal levels of 15-F_{2t}-IsoP-M in rats are readily quantifiable. Interestingly, administration of CCl_4 was associated with a 24-fold increase in the excretion of 15-F_{2t}-IsoP-M (Fig. 6).

DISCUSSION

Because oxidant stress is believed to play an important role in the pathophysiology of a number of human diseases, the development of methods to accurately assess oxidative injury *in vivo* are extremely important. We have previously utilized quantification of the F_2-IsoPs to examine oxidant stress in animal and human biological fluids and tissues (8). Despite the utility of measuring these compounds, several potential problems exist. First, artifactual generation of F_2-IsoPs can occur in biological specimens, particularly plasma, if they are improperly handled. Second, quantification of F_2-IsoPs in biological tissues or fluids may represent IsoP production locally in a particular organ rather than providing a systemic index of oxidative injury. In this regard, several research groups have suggested that quantification of urinary F_2-IsoPs is an accurate index of total body oxidant status (17, 18). On the other hand, analogous to cyclooxygenase-derived PGs, it is reasonable to assume that the majority of urinary F_2-IsoPs may derive from local production in the kidney (8, 19). Thus the development of methods to assess a urinary metabolite of IsoPs is likely to provide a more accurate index of systemic oxidant stress.

Having previously identified the major urinary metabolite of 15-F_{2t}-IsoP in humans (11), we have subse-

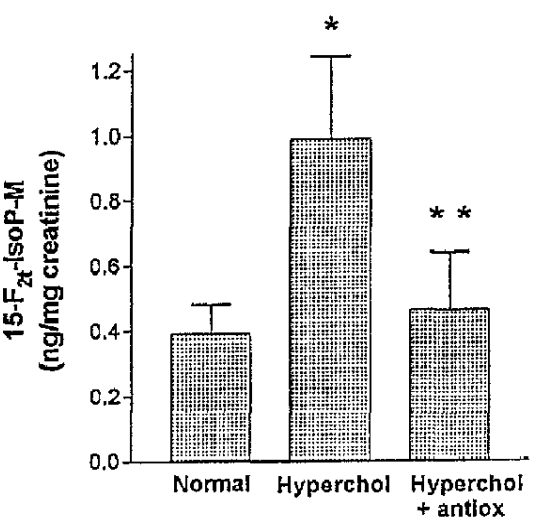

FIG. 5. Levels of urinary 15-F_{2t}-IsoP-M in normal human volunteers ($n = 10$), individuals with polygenic hypercholesterolemia ($n = 5$), and individuals with polygenic hypercholesterolemia after treatment with antioxidants for 8 weeks ($n = 5$). *$P < 0.05$, metabolite levels in normals versus hypercholesterolemics; **$P < 0.05$, hypercholesterolemics versus hypercholesterolemics treated with antioxidants.

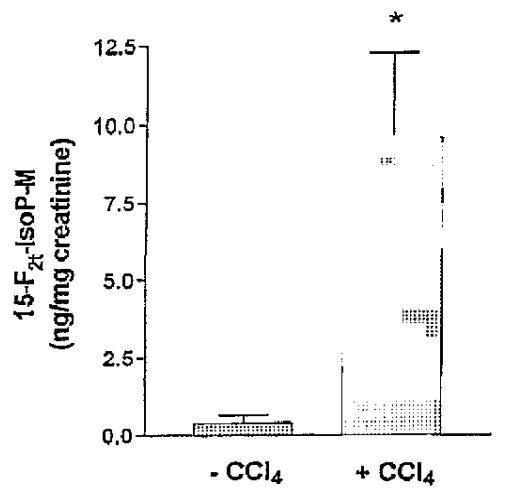

FIG. 6. Levels of urinary 15-F_{2t}-IsoP-M in rats at baseline and after treatment with CCl_4 (1 ml/kg). *$P < 0.05$, $-CCl_4$ versus $+CCl_4$.

quently developed methods to assess the *in vivo* generation of this compound employing GC/NICI MS. The assay that has been developed is both highly sensitive and accurate. As illustrated in Fig. 3, the starred (*) peak in the *m/z* 543 chromatogram that we quantify readily separates on GC from material eluting several seconds later. Based on previously discussed experimental evidence, the additional peaks in the *m/z* 543 chromatogram likely represent metabolites of other endogenously derived F_2-IsoPs. Further support for this comes from the fact that their levels increase in parallel with 15-F_{2t}-IsoP-M in settings of oxidative injury (data not shown).

We have found that levels of 15-F_{2t}-IsoP-M can be decreased in humans by antioxidants. These observations are highly relevant in light of the fact that a number of clinical trials are either planned or underway to assess the role of antioxidants in disease prevention (20). Having now developed an accurate noninvasive tool to assess oxidant stress status *in vivo*, it would be highly informative to correlate disease outcome with IsoP formation in these studies.

Although GC/MS assays for eicosanoids are generally not as efficient as other methods of analysis (i.e., immunoassays), approximately 15 to 20 samples can be processed and analyzed for 15-F_{2t}-IsoP-M per day using this method. In this regard, it is as equally facile as other GC/MS assays reported for the quantification of other prostanoids.

In summary, we have reported methods to accurately quantify the major urinary metabolite of the IsoP 15-F_{2t}-IsoP which is produced in abundance *in vivo*. It is anticipated that this mass spectrometric assay will prove to be a valuable analytical tool to further explore the role of oxidant stress and IsoP formation in the pathophysiology of human disease.

ACKNOWLEDGMENTS

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